REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS AND AMENDMENTS

Claims 1-52 were pending in this application when last examined.

Claims 1-3, 14, 15 and 36-46 were examined on the merits and stand rejected.

Claims 4-13, 16-35 and 47-52 were withdrawn as non-elected subject matter.

Claims 37, 39, 41, 43, 45 and 46 were objected to.

The Title, Abstract and Specification are amended to clarify the invention.

Claims 37, 39, 41, 43, 45 and 46 are cancelled without prejudice or disclaimer thereto. Applicants reserve the right to file a Continuation or Divisional Application on any cancelled subject matter.

Claims 36, 38, 40, 42 and 44 are amended to recite "1,2,5-oxadiazolo[3,4-a]1,2,5-oxadiazolo[3,4-e]1,2,5-oxadiazolo[3,4-i]1,2,5-oxadiazolo[3,4-m][16]annulene". Support for this amendment can be found on page 70, lines 3-13 and page 78, lines 12-19, of the specification as filed.

Claims 7 and 15 is amended to clarify the claimed invention.

Claim 14 is also amended. Support can be found on page 20, line 16 to page 21, line 4, of the specification as filed.

No new matter has been added.

II. FOREIGN PRIORITY

The Examiner is respectfully requested to fully acknowledge the claim for foreign priority by checking boxes 12(a)(1, 2, or 3) on the coversheet of the next response. Applicants note that certified copies of the priority documents were submitted to the PTO by the International Bureau as evidenced by the enclosed Form PCT/IB/304.

III. OBJECTIONS TO THE SPECIFICATION

In item 2 on pages 2-3 of the Office Action, the Title, Abstract and Specification were objected to for the noted reasons. Herein are amendments to alleviate these objections.

No new matter has been added by these amendments.

IV. CLAIM OBJECTION

In item 3 on page 3 of the Office Action, claims 37, 39, 41, 43, 45 and 46 were objected to for the noted reasons. This objection is moot as these claims are cancelled to expedite allowance.

V. ENABLEMENT REJECTIONS

In item 4A on pages 3–4 of the Office Action, claims 1-3 were rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification is only enabling for stimulating feeding, increasing body weight and increasing epididymal fat in male Wistar rats and not for a method of stimulating feeding, increasing body weight, or increasing any other type of fat in any other mammals.

In item 4B on pages 4-5, claims 14 and 15 were rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification is only enabling for stimulating feeding, increasing body weight and increasing epididymal fat in male Wistar rats and not for a method of treating any and all diseases which require body weight gain.

Finally, in item 4C on pages 4-5, claims 14 and 15 were rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification is only enabling for stimulating feeding, increasing body weight and increasing epididymal fat in male Wistar rats and not for a method of reducing these effects, or decreasing any other type of fat weight in any other mammals.

Applicants respectfully traverse these rejections as applied to the remaining amended claims.

In regard to items 4A, 4B and 4C, attached herewith is an article (Attachment A: Brown et al., "Sibutramine reduces feeding, body fat and improves insulin resistance in dietary-obese male Wistar rats independently of hypothalamic neuropeptide Y", Journal of Pharmacology, (2001), 132: 1898-1904) showing that male Wistar rats are a model of dietary obesity. Please see items 1 and 2 of the Abstract. Furthermore, such model was used to show that Sibutramine

decreases feeding, body fat and improves insulin resistance. Applicants note that Sibutramine was approved by the FDA in 1997 for the treatment of obesity in humans.

Thus, Applicants submit that this article shows that male Wistar rats are a well-known art-accepted model for obesity as of the priority date of this application.

In items 4A, 4B and 4C, the Office was of the position that the model used in the specification is not enabling for the scope of the claims. In light of this publication and the FDA approval of Sibutramine for anti-obesity in humans, Applicants believe such position is untenable and therefore these rejections should be withdrawn.

Further, in regard to the rejection in item 4B, it is noted that claims 14 and 15 are amended to expedite allowance. In particular, claim 14 has been limited to a method for recovering feeding and/or body weight gain in a patient having a disease involving reduced feeding and/or weight loss. Further, claim 15 has been limited to anorexia or cachexia which was indicated as enabled by the Examiner. Thus, Applicants suggest that this rejection is untenable.

Finally, with regard to item 4C of the Office Action, Applicants note that the claims have been limited to a particular compound as disclosed in the specification. Thus, there is no longer a "reach-through" issue. Thus, Applicants note that the enclosed reference shows that male Wistar rats are an acceptable model for anti-obesity and that the claims have been limited to known compound. Thus, this rejection is untenable and should be withdrawn.

VI. WRITTEN DESCRIPTION REJECTION

In item 5 on page 6 of the Office Action, claims 37, 39, 41, 43, 45 and 46 were rejected. These claims have been cancelled to expedite allowance and therefore this rejection is moot.

VII. INDEFINITENESS REJECTION

On page 6 of the Office Action, claims 36 and 46 were rejected under 35 U.S.C. § 112, second paragraph, for indefiniteness for the acronym "SALPR". This rejection is overcome for reasons which are self-evident.

CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

Takayuki HIDA et al.

By: _____

William R. Schmidt, II
Registration No. 58,327
Attorney for Applicants

WRS/lc
Washington, D.C. 20006-1021
Telephone (202) 721-8200
Facsimile (202) 721-8250
March 17, 2009

British Journal of Pharmacology (2001) 132, 1898–1904

www.nature.com/bjp

Sibutramine reduces feeding, body fat and improves insulin resistance in dietary-obese male Wistar rats independently of hypothalamic neuropeptide Y

[1]Michael Brown, [1]Chen Bing, [1]Peter King, [1]Lucy Pickavance, [2]David Heal & *,[1]John Wilding

[1]Department of Medicine, University of Liverpool, PO Box 147, Liverpool, L69 3GA and [2]Knoll Pharmaceuticals Research and Development, Nottingham, NG1 1GF

1 We studied the effects of the novel noradrenaline and serotonin (5-HT) reuptake inhibitor sibutramine on feeding and body weight in a rat model of dietary obesity, and whether it interacts with hypothalamic neuropeptide Y (NPY) neurones.

2 Chow-fed and dietary-obese (DIO) male Wistar rats were given sibutramine (3 mg kg $^{-1}$ day $^{-1}$ p.o.) or deionized water for 21 days.

3 Sibutramine decreased food intake throughout the treatment period in both dietary-obese rats ($P<0.0001$) and lean rats ($P<0.0001$). Weight gain was reduced so that final body weight was 10% lower in dietary-obese ($P<0.005$) and 8% lower in lean ($P<0.05$) rats *versus* their untreated controls. Plasma leptin concentration was lower in sibutramine-treated dietary-obese rats ($P<0.05$), and in treated lean rats ($P<0.05$). Using the homeostasis model assessment (HOMA) as a measure of insulin resistance, untreated DIO rats were significantly more insulin resistant than controls ($P<0.005$), and this was corrected by sibutramine treatment ($P<0.05$). Neither hypothalamic NPY mRNA nor NPY peptide levels in a number of hypothalamic nuclei were significantly altered by sibutramine compared to untreated controls.

4 The hypophagic and anti-obesity effects of sibutramine in dietary-obese Wistar rats appear not to be mediated by inhibition of ARC NPY neurones.

British Journal of Pharmacology (2001) 132, 1898–1904

Keywords: Obesity; sibutramine; HOMA; leptin; neuropeptide Y

Abbreviations: ARC, arcuate nucleus with median eminence; DMN, dorsomedial nucleus; HOMA, homeostatic model assessment; LHA, lateral hypothalamic area; MPO, medial preoptic area; NPY, neuropeptide Y; PVN, paraventricular nucleus; VMN, ventromedial nucleus

Introduction

Sibutramine (BTS 54 524; N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine hydrochloride monohydrate) is a novel 5-HT and noradrenaline reuptake inhibitor (SNRI) anti-obesity drug (Stock, 1997), that has recently been licensed for the treatment of obesity in several countries. The mechanisms of sibutramine-induced weight loss are thought to include enhancement of satiety (Halford *et al.*, 1998) and an increase in thermogenesis (Connoley *et al.*, 1999; Hansen *et al.*, 1998; McNeely & Goa, 1998). Sibutramine markedly reduces feeding behaviour and is effective in inducing weight loss in lean and genetically obese rodents (Stricker-Kongrad *et al.*, 1995). Moreover, it improves glucose tolerance and decreases plasma insulin levels in these animals, implying that sibutramine may improve insulin sensitivity (Day & Bailey, 1998).

Pre-treatment with 5-HT or noradrenaline antagonists can partially or completely reverse the hypophagic effect of sibutramine, indicating that both neurotransmitters are involved in its pharmacological actions. In addition, fluoxetine and nisoxetine, which are selective reuptake inhibitors of 5-HT and noradrenaline respectively, have no effect on food intake when given alone, but they profoundly inhibit food intake when given in combination (equivalent to sibutramine's action), demonstrating a synergistic interaction of those two monoamines in the control of ingestive behaviour (Jackson *et al.*, 1997a). The use of selective monoamine antagonists has confirmed that the acute satiety-inducing effects of sibutramine involves α_1 and β_1 adrenoceptors, as well as $5HT_c$ and possibly $5HT_{1A}$ receptors (Jackson *et al.*, 1997b). Since neither sibutramine nor its two metabolites exhibit affinity for α_1, β_1 or 5HT receptors, the drug appears to enhance monoaminergic function by inhibiting noradrenaline and 5HT uptake (Heal *et al.*, 1998).

5-HT acts on the hypothalamus to cause anorexia, weight loss (Blundell *et al.*, 1995) and increased thermogenesis (Le Feuvre *et al.*, 1991). A large number of peptides and other neurotransmitters found in the hypothalamus have effects on energy balance, including neuropeptide Y (NPY), galanin, melanocyte stimulating hormone (*via* the melanocortin-4 receptor) and recently cocaine and amphetamine-related transcript (CART) (Williams *et al.*, 2000). Evidence exists to suggest that the anti-obesity actions of serotonin may be mediated by inhibition of hypothalamic neurones that express the powerful appetite-stimulating peptide NPY (Dryden *et al.*, 1996; Rogers *et al.*, 1991), and this study therefore

*Author for correspondence; E-mail: J.P.H.Wilding@liv.ac.uk

Attachment A

focused on the possible involvement of NPY in mediating the effects of sibutramine on energy balance.

NPY is synthesized in arcuate nucleus (ARC) neurones that project mainly to the hypothalamic paraventricular nucleus (PVN) and dorsomedial nucleus (DMN) (Chronwall *et al.*, 1985; Morris, 1989). These two latter sites are important integrating centres in the control of energy homeostasis, and are closely related to the ventromedial nucleus (VMN) and other sites connected with the sympathetic and parasympathetic nuclei of the brain stem (Sawchenko *et al.*, 1985). NPY injected into the PVN induces insatiable hyperphagia (Stanley *et al.*, 1986) and decreases whole-body energy expenditure by reducing the sympathetic stimulation of thermogenesis in brown adipose tissue (BAT) (Billington *et al.*, 1994; Walker & Romsos, 1993); its repeated administration leads to obesity with hyperinsulinemia and marked insulin resistance (Zarjevski *et al.*, 1994). Postulated peripheral signals regulating the activity of NPY hypothalamic neurons include insulin (Schwartz *et al.*, 1992) and leptin (Vuagnat *et al.*, 1998; Wang *et al.*, 1997). Centrally, serotonergic neurones in the raphe nucleus project to the hypothalamus and terminate around the NPY cell bodies in the arcuate nucleus (ARC) of the hypothalamus, and NPY-immunoreactive endings in the paraventricular nucleus (PVN) (Pelletier, 1990). Previous studies have suggested that some agents that mimic or enhance 5-HT action and which cause hypophagia and weight loss also inhibit NPY neuronal activity (Dryden *et al.*, 1996), although this is not the case for all such agents (Wilding *et al.*, 1992a). Conversely, drugs that block 5-HT release or antagonize its action at 5-HT_{1B} and -C receptors stimulate feeding and NPY neuronal activity (Dryden *et al.*, 1995). This implies that serotonin may tonically inhibit the NPY neurones, and that the latter may mediate the effects of serotonin on energy homeostasis.

Unlike in rat models the majority of human obesity is polygenic, and is due to difficulty in regulating intake in the face of an increased availability of highly palatable foods and a decline in physical activity (Andersson, 1996). Rats fed a highly palatable diet develop moderate obesity, which like most human obesity is associated (at least in younger animals), with increased food intake, increased thermogenesis and insulin resistance (Stock & Rothwell, 1986). We therefore chose this model for our studies which were designed to answer the following questions, as to whether sibutramine is effective at reducing body weight in DIO, and secondly whether sibutramine improved insulin resistance in this model and finally whether the effects of sibutramine were mediated in part by modulation of hypothalamic NPY neurons.

Methods

Animals

Ninety-six male Wistar rats (150–200 g; A. Tuck and Sons, Cambridge, U.K.), were randomly allocated into two groups each containing 48 rats, and housed individually in wire-bottomed cages in a room maintained at 22°C, on reversed phase lighting 12 h dark:light cycle (lights on at 0300 h). Forty-eight rats were fed a highly palatable diet for 12 weeks which consisted of 33% chow (CRM, Biosure, Cambridge, U.K.) 33% Nestlé milk (Nestlé, U.K.), 7% sucrose (Tate & Lyle, U.K.) and 27% tap water by weight. This diet produces reliable weight gain over controls, providing 68% of energy as carbohydrate, 16% as protein and 13% as fat (Wilding *et al.*, 1992b). The remaining 48 animals had free access to standard pelleted chow (CRM, Biosure, Cambridge, U.K.), which provides 76.8% of energy as carbohydrate, 19.2% as protein and 4.3% as fat.

Treatment procedures

After the 12 weeks of dietary manipulation, 16 animals in each group were either given sibutramine, pair-fed to match the intake of sibutramine-treated animals or treated with vehicle alone. Sibutramine HCl was synthesized at Knoll Pharmaceuticals (Nottingham, U.K.) and was dissolved in deionized water, dose volume 1 ml kg^{-1}, and was administered at 1200 h daily at a dose of 3 mg kg^{-1} day^{-1} p.o. by gavage for a period of 21 days. This dose has previously been shown to be effective at reducing food intake in the rat and reduces food intake by 50% over a 2 h period (Jackson *et al.*, 1997a). Lean and dietary-obese controls similarly received an equal volume of deionized water. Body weight and food intake were measured immediately before drug administration each day throughout the treatment period.

At the end of the study, rats were killed by carbon dioxide inhalation and exsanguinated immediately by cardiac puncture. Plasma was stored at −40°C for subsequent measurement of leptin, insulin and glucose concentrations. Epididymal and perirenal white adipose tissue along with gastrocnemius muscle was dissected and weighed, to allow calculation of fat/lean ratio. All animal procedures were carried out in accordance with U.K. Home Office regulations, under the authority of the relevant project licence.

Hypothalamic microdissection

For measurement of NPY mRNA, ($n=8$ animals per group), a block of mediobasal hypothalamic tissue was dissected from a frontal brain slice cut between the middle optic chiasm and the mammillary bodies. The block extended laterally to the perihypothalamic nucleus and superiorly to the anterior commissure; the ARC is the only site containing significant NPY mRNA levels within these boundaries. The tissue was immediately snap-frozen in liquid nitrogen and stored at −80°C until subsequent extraction of RNA.

For measurement of regional hypothalamic NPY levels, ($n=8$ animals per group), the brain was rapidly removed and a block, including the hypothalamus, was removed by vertical cuts 1 mm anterior to the optic chiasm and immediately posterior to the mammillary bodies. Subsequently eight frontal slices of 350–500 μm were then cut from the tissue block, using a vibrating microtome, as previously described (Bing *et al.*, 1999). The following six selected areas were microdissected, by punching out with a blunt 18-gauge needle: PVN, MPO, VMH, DMN, LHA and ARC (including the median eminence). The tissue from each area in each rat was pooled and boiled for 10 min in 400 μl of 0.1 M hydrochloric acid, and the samples were sonicated for 30 s to disperse the tissue and extract NPY. The extracts were

frozen at −40°C until assayed for NPY and protein concentrations.

Assays

Regional hypothalamic NPY concentrations were measured using an in-house radioimmunoassay (RIA) which employed ^{125}I-labelled porcine NPY (pNPY; Amersham, Buckinghamshire, U.K.) and pNPY as standard (Bachem Inc, Essex, U.K.). NPY antiserum raised in our laboratory in a rabbit against porcine NPY, was used in a final dilution of 1:90,000. The sensitivity of the assay was 2 fmol per tube, with an intra-assay coefficient of variation (CV) <4.0%. Samples were measured in duplicate in a single assay. Protein concentrations in hypothalamic extracts were determined by a BCA-200 protein assay kit, (Pierce, IL, U.S.A.) and NPY levels in each region were expressed as fmol per μg protein.

Plasma leptin levels were determined using a RIA kit from Linco Research (Biogenesis, Poole, Dorset, U.K.), with an intra-assay CV of 3%. Plasma insulin was measured using an enzyme-linked immunosorbant assay (ELISA) kit (IDS, Boldon, Newcastle-upon-Tyne, U.K.) and had a within-assay CV of <6%. Plasma glucose was measured using a glucose-oxidase based system (Roche Diagnostics, U.K.).

Measurements of NPY mRNA

Total hypothalamic RNA was isolated from hypothalamic tissue blocks using the guanidinium thiocyanate phenol-chloroform method and RNA concentration determined from the absorbance at 260 nm. Twenty micrograms of total RNA per sample was applied to a 1% agarose-formaldehyde gel and separated by electrophoresis. The RNA was transferred overnight to a neutral-charged membrane (Schleicher and Schuell, London, U.K.) by capillary blotting and then cross-linked under u.v. light.

Pre-hybridization was performed at 42°C for 1 h in a buffer containing 50% formamide, 5×SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% N-lauroylsarcosine, 50 mM sodium phosphate (pH 7.0) and 7% SDS. Hybridization was at 42°C overnight in the pre-hybridization solution with a 42-mer oligonucleotide (R&D Systems, Oxon, U.K.) which was end-labelled (3′ and 5′) with digoxigenin at a concentration of 25 ng ml^{-1}. Post-hybridization washes were performed as described previously (Trayhurn & Duncan, 1994). The membrane was incubated with an antibody against digoxigenin (Fab fragment; Boehringer), which was conjugated to alkaline phosphatase, for 30 min at room temperature. The membrane was then sprayed with 0.25 mM chemiluminescence substrate CDP-star (Tropix, MA, U.S.A.). Signals were obtained by exposure of the membrane to X-ray film for 30 min at room temperature, and the 0.9 kb band corresponding to NPY mRNA was quantified using image-scan densitometry (AIS System, Imaging Technology, Brock University, St Catherines, Ontario, Canada).

To check the loading and transfer of RNA during blotting, the blot was stripped and re-probed for 18S rRNA with a 31-mer digoxigenin-labelled antisense oligonucleotide at a concentration of 10 pg/ml, as previously described (Trayhurn *et al.*, 1995). The amount of NPY mRNA was expressed as the NPY mRNA/18S rRNA ratio.

Statistical analyses

Data are expressed as mean±s.e.mean. Blood analytes, NPY mRNA levels were compared between groups using one-way analysis of variance (ANOVA). For food intake, body weight and NPY concentrations in individual hypothalamic regions, two-way ANOVA coupled to a Bonferonni *post-hoc* modified *t*-test was performed to determine whether there were significant differences between groups. Group differences in NPY levels within individual nuclei were then further examined by Student's unpaired *t*-test. A P value of 0.05 or less at two-tail level was taken as significant.

Results

Effect of highly palatable diet upon body weight

Animals fed the palatable diet gained weight more rapidly than controls, and despite starting at similar weights were 18.6% heavier at the end of the 12-week period of dietary manipulation (535±6 g *vs* 451±7 g; $P<0.0001$).

Food intake and body weight changes

Sibutramine treatment for 21 days significantly reduced food intake and body weight in both dietary-obese and chow-fed rats, the effects being more marked in the dietary-obese group. Dietary-obese animals given sibutramine showed a rapid-onset (from day 2) suppression of food intake compared with the vehicle-treated controls, that lasted throughout the experimental period ($P<0.0001$) (Figure 1a). Lean rats treated with sibutramine also ate less than the controls from day 3, with a less marked reduction in total food intake ($P<0.0001$) (Figure 1b).

Sibutramine caused a progressive and significant decline in weight gain from day 2 in dietary-obese rats ($P<0.0001$), with their final body weight being 9% lower ($P<0.005$) than DIO controls (Figure 2). Lesser decreases in weight gain were also seen in treated lean rats ($P<0.05$), with final body weight being 7% below lean controls (Figure 2).

Fat/lean ratio was 51% higher in untreated dietary-obese rats than in chow-fed controls ($P<0.0001$) (Figure 3). Sibutramine treatment led to a significant reduction in the lean/fat ratio of 23.7% ($P=<0.01$) in dietary-obese but not in pair-fed dietary-obese or chow-fed animals (Figure 3).

Plasma leptin concentrations and HOMA analysis

Plasma leptin levels were 3 fold higher ($P<0.01$) in untreated dietary-obese than in lean animals (Figure 4). Sibutramine caused a 63% fall in leptin levels in dietary-obese ($P<0.05$), and a 44% fall in lean rats ($P<0.05$) (Figure 4).

Untreated dietary-obese rats were significantly more insulin resistant than chow-fed control animals [HOMA 0.47±0.10 (diet) *vs* 0.27±0.03 (controls); $P<0.005$], and this was corrected by treatment with sibutramine [HOMA 0.21±0.03; $P<0.05$ *vs* diet+vehicle], however pair-feeding dietary-obese animals only showed a small, non-significant reversal of insulin resistance (Figure 5).

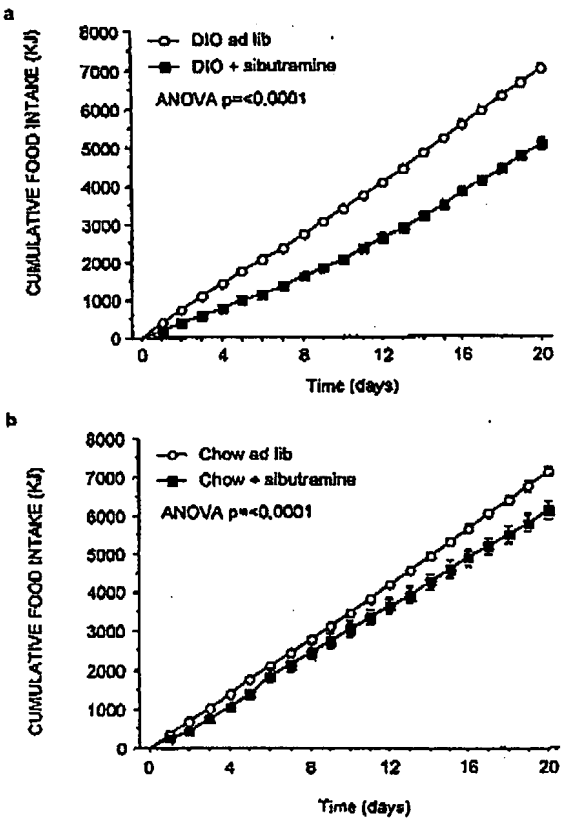

Figure 1 Cumulative food intake in DIO (a) and chow (b) rats treated orally with sibutramine (3 mg kg $^{-1}$ day $^{-1}$ p.o.) or vehicle for 21 days. Show are DIO controls ($n=16$), DIO treated with sibutramine ($n=16$), chow controls ($n=16$) and chow treated with sibutramine ($n=16$) (means±s.e.mean). Total cumulative food intake for both experimental groups is shown in (c) ($n=16$) (means±s.e.mean).

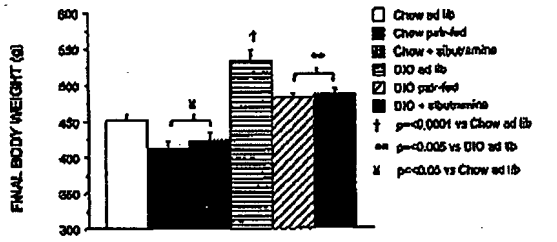

Figure 2 Final body weight of DIO and chow-fed rats treated orally with sibutramine (3 mg kg $^{-1}$ day $^{-1}$ p.o.) or vehicle for 21 days ($n=16$ per group; means±s.e.mean).

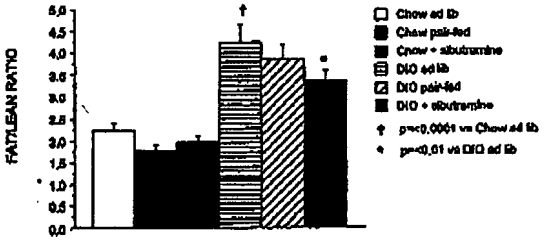

Figure 3 Fat/lean ratio of DIO and chow/fed rats treated orally with sibutramine (3 mg kg $^{-1}$ day $^{-1}$ p.o.) or vehicle for 21 days ($n=16$ per group; means±s.e.mean).

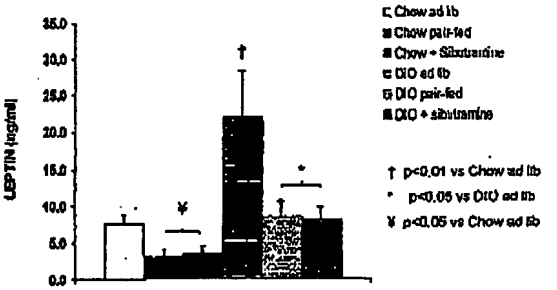

Figure 4 Plasma leptin levels of DIO and chow-fed rats treated orally with sibutramine (3 mg kg $^{-1}$ day $^{-1}$ p.o.) or vehicle for 21 days ($n=12$ per group; means±s.e.mean).

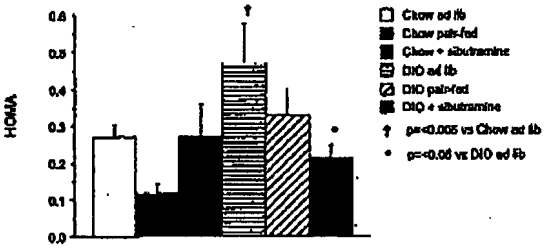

Figure 5 HOMA index of DIO and chow-fed rats treated orally with sibutramine (3 mg kg $^{-1}$ day $^{-1}$ p.o.) or vehicle for 21 days ($n=12$ per group; means±s.e.mean).

Hypothalamic NPY and NPY mRNA levels

Hypothalamic NPY mRNA levels were unchanged in dietary-obese rats compared to chow-fed rats ($P=$ns) (Table 1). There were no significant changes in NPY mRNA levels associated with sibutramine treatment or pair-feeding of animals (Table 1).

NPY concentrations in the six hypothalamic regions in dietary-obese and chow-fed control rats treated with

Table 1 Changes in total hypothalamic NPY mRNA and concentrations of NPY peptide in individual hypothalamic nuclei after dietary manipulation and sibutramine treatment

	Chow ad libitum	*Chow pair-fed*	*Chow and sibutramine*	*Diet* ad libitum	*Diet pair-fed*	*Diet and sibutramine*
NPY mRNA: 18S rRNA ratio	0.89±0.21	0.21±0.06	0.11±0.03	0.57±0.12	0.91±0.57	0.27±0.04
NPY level (fmol/μg protein)						
MPO	3.51±0.42	3.44±0.28	2.73±0.42	2.76±0.28	2.77±0.50	2.82±0.45
PVN	4.82±0.67	4.44±0.59	4.63±0.46	4.91±0.51	4.46±0.76	5.50±1.03
VMN	1.92±0.12	2.26±0.23	2.12±0.33	2.41±0.28	1.71±0.23	1.96±0.61
DMN	2.98±0.40	2.86±0.17	2.42±0.31	2.33±0.20	2.83±0.67	3.22±0.35
ARC	2.19±0.14	2.45±0.21	1.93±0.23	1.74±0.19	1.64±0.18	1.93±0.23
LHA	1.05±0.18	1.40±0.14	1.20±0.20	1.37±0.09	1.04±0.20	1.63±0.22

Data are mean±s.e.mean ($n=8$). MPO, medial preoptic area; PVN, paraventricular nucleus; VMN, ventromedial nucleus; DMN, dorsomedial nucleus; ARC, arcuate nucleus with median eminence; LHA, lateral hypothalamic area.

sibutramine ($n=8$) or deionized ($n=8$), or pair-fed ($n=8$) for 21 days are shown in Table 1. Two-way ANOVA revealed that there was no significant effects attributable to group. Neither treatment with sibutramine or pair-feeding had any significant effect on NPY concentrations within individual hypothalamic nuclei (Table 1).

Discussion

Our results show that sibutramine significantly attenuates food intake and body weight gain in dietary-obese Wistar rats. Furthermore, this weight loss is accompanied by depletion of body fat stores. Sibutramine treatment also ameliorates the insulin resistance seen in this model of obesity. However, in contrast to the serotonin-releasing agents previously studied (Dryden *et al.*, 1996; Rogers *et al.*, 1991), sibutramine does not appear to mediate these effects by altering hypothalamic NPY.

We used an oral dose of sibutramine (3 mg kg^{-1} day^{-1} p.o.), which caused a reduction in both food intake and body weight gain in dietary obese rats. These data agree with a previous study (Fantino *et al.*, 1995). In agreement with studies in obese humans, body compositional changes induced by sibutramine were reflected by depletion of adipose tissue rather than lean tissue mass (Griffiths *et al.*, 1995). This effect was reflected by a concomitant fall in plasma leptin levels, which was proportional in both chow fed and dietary obese groups to the reduced fat mass, consistent with leptin's correlation with body fat mass in rodents and humans (Considine *et al.*, 1996; Maffei *et al.*, 1995; Solin *et al.*, 1997).

Consistent with the known association between obesity, insulin resistance and diabetes, the weight loss in the sibutramine dietary obese group was associated with a fall in the HOMA index, indicating an amelioration of insulin resistance. Insulin resistance was improved to a greater extent with sibutramine treatment compared with the pair-fed dietary obese group, suggesting that sibutramine might have an independent effect to improve insulin sensitivity, beyond those effects that are due to weight loss. This could be related to the selective loss of adipose tissue seen with sibutramine treatment, as adiposity is an important determinant of insulin sensitivity (Jung, 1997) or a direct effect as has been recently reported (Day & Bailey, 1998).

We have found that sibutramine at a dose sufficient to induce and sustain hypophagia and weight loss in dietary obese rats, does not appear to be associated with changes in regional hypothalamic NPY concentrations. In addition, we have not shown any changes in total hypothalamic NPY mRNA with this treatment. Our findings are consistent with previous reports that hypothalamic NPY mRNA levels in dietary obese rats are not significantly altered after a 6 week exposure to the diet (Wilding *et al.*, 1992a,b). However, these studies did find increases in NPY concentrations in the arcuate and paraventricular nuclei. There are several possible reasons for the lack of change in hypothalamic NPY peptide content in this study. Hypothalamic NPY release rates could be altered by dietary obesity and sibutramine, without altering mRNA or peptide content, however, release rates when measured by microdialysis are consistent with what would be expected from measurement of mRNA levels and peptide content (Lambert *et al.*, 1994), so we consider this possibility unlikely. The DIO animals were fed the diet for 15 weeks, compared to 6–7 weeks in the previously reported studies. It is possible that changes in NPY concentration in the ARC and PVN are transient, representing a metamorphosis between the preobese to the fully obese DIO rat, where it is seen that a number of brain systems appear to normalize with the appearance of obesity. These include α_2-adrenoreceptors (Levin, 1990; Wilmot *et al.*, 1988), noradrenergic (Levine *et al.*, 1995) and hypothalamic neuronal function (Levin, 1991; Levin & Sullivan, 1989).

Recent evidence suggests that preobese DIO-prone rats have higher arcuate NPY mRNA levels than dietary resistant rats before exposure to a high energy diet, however the elevated NPY levels are reduced after 12 weeks of high energy-diet intake (Levin, 1999). This may account for the results seen in this study, where DIO rats exhibited no change in hypothalamic NPY after 15 weeks on the diet. This would suggest that sibutramine acts independently of the NPYergic system. Interestingly, NPY levels and mRNA was unaffected by pair-feeding. However, towards the last 4 days of the study, food intake between the sibutramine treated groups and untreated *ad libitum* groups was not significantly different. This means that the pair-fed groups received similar amounts of food to the untreated *ad libitum* groups, and were not under conditions that would invoke up-regulation of hypothalamic NPY normally seen in food restricted rodents (Abizaid *et al.*, 1997; Brady *et al.*, 1990; Dryden *et al.*, 1996; Lewis *et al.*, 1993).

The hypothalamic control of appetite is complex, and different components have specific functions in regulating

satiety, hunger, food choice and energy expenditure, with a significant degree of redundancy, for example the NPY knockout mouse gains body weight normally (Williams *et al.*, 2000). It is therefore possible that sibutramine could alter food intake and or energy expenditure by influencing one or more other hypothalamic peptidergic and non-peptidergic neurotransmitter systems that are involved in the control of energy balance, but were not assessed in the studies described here. Further studies will however be need to answer these questions.

In conclusion, we have shown that sibutramine induces hypophagia and slows weight gain in DIO and chow-fed Wistar rats. These changes were accompanied by preferential loss of body fat stores and improved insulin sensitivity. Furthermore, the data presented here would suggest that sibutramine acts independently of the NPYergic system.

References

ABIZAID, A., WALKER, C.D. & WOODSIDE, B. (1997). Changes in neuropeptide Y immunoreactivity in the arcuate nucleus during and after food restriction in lactating rats. *Brain Res.*, **761**, 306–312.

ANDERSSON, L.B. (1996). Genes and obesity [editorial]. *Ann. Med.*, **28**, 5–7.

BILLINGTON, C.J., BRIGGS, J.E., HARKER, S., GRACE, M. & LEVINE, A.S. (1994). Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. *Am. J. Physiol.*, **266**, R1765–R1770.

BING, C., KING, P., PICKAVANCE, L., BROWN, M., ZIEGLER, D., KAAN, E. & WILLIAMS, G. (1999). The effect of moxonidine on feeding and body fat in obese Zucker rats: role of hypothalamic NPY neurones. *Br. J. Pharmacol.*, **127**, 35–42.

BLUNDELL, J.E., LAWTON, C.L. & HALFORD, J.C. (1995). Serotonin, eating behavior, and fat intake. *Obes. Res.*, **3** (Suppl 4): 471S–476S.

BRADY, L.S., SMITH, M.A., GOLD, P.W., & HERKENHAM, M. (1990). Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology*, **52**, 441–447.

CHRONWALL, B.M., DIMAGGIO, D.A., MASSARI, V.J., PICKEL, V.M., RUGGIERO, D.A. & O'DONOHUE, T.L. (1985). The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience*, **15**, 1159–1181.

CONNOLEY, I.P., LIU, Y.L., FROST, I., RECKLESS, I.P., HEAL, D.J. & STOCK, M.J. (1999). Thermogenic effects of sibutramine and its metabolites. *Br. J. Pharmacol.*, **126**, 1487–1495.

CONSIDINE, R.V., SINHA, M.K., HEIMAN, M.L., KRIAUCIUNAS, A., STEPHENS, T.W., NYCE, M.R., OHANNESIAN, J.P., MARCO, C.C., MCKEE, L.J., BAUER, T.L. & CARO, J.F. (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans [see comments]. *N. Engl. J. Med.*, **334**, 292–295.

DAY, C. & BAILEY, C.J. (1998). Effect of the antiobesity agent sibutramine in obese-diabetic ob/ob mice. *Int. J. Obes. Relat. Metab. Disord.*, **22**, 619–623.

DRYDEN, S., FRANKISH, H.M., WANG, Q., PICKAVANCE, L. & WILLIAMS, G. (1996). The serotonergic agent fluoxetine reduces neuropeptide Y levels and neuropeptide Y secretion in the hypothalamus of lean and obese rats. *Neuroscience*, **72**, 557–566.

DRYDEN, S., PICKAVANCE, L., FRANKISH, H.M. & WILLIAMS, G. (1995). Increased neuropeptide Y secretion in the hypothalamic paraventricular nucleus of obese (fa/fa) Zucker rats. *Brain Res.*, **690**, 185–188.

FANTINO, M., MARTEL, P., SOUQUET, A.M., WIETESKA, L., BRONDEL, L., & COURCIER, S. (1995). Decrease of food intake and weight loss induced by sibutramine in the rat. *Obes. Res.*, **3** (Suppl 4): 628S.

GRIFFITHS, J., BLOOM, S.R., FINER, N., BANKS, L.M. & ROMANAC, F.M. (1995). Body compositional changes following weight loss induced by sibutramine. *Int. J. Obes. Relat. Metab. Disord.*, **19** (Suppl 2): 144.

HALFORD, J.C.G., WANNINAYAKE, S.C.D. & BLUNDELL, J.E. (1998). Behavioural satiety sequence (BSS) for the diagnosis of drug action on food intake. *Pharmacol. Biochem. Behav.*, **61**, 159–168.

HANSEN, D.L., TOUBRO, S., STOCK, M.J., MACDONALD, I.A. & ASTRUP, A. (1998). Thermogenic effects of sibutramine in humans. *Am. J. Clin. Nutr.*, **68**, 1180–1186.

HEAL, D.J., CHEETHAM, S.C., PROW, M.R., MARTIN, K.F. & BUCKETT, W.R. (1998). A comparison of the effects on central 5-HT function of sibutramine hydrochloride and other weight-modifying agents. *Br. J. Pharmacol.*, **125**, 301–308.

JACKSON, H.C., BEARHAM, M.C., HUTCHINS, L.J., MAZURKIEWICZ, S.E., NEEDHAM, A.M. & HEAL, D.J. (1997b). Investigation of the mechanisms underlying the hypophagic effects of the 5-HT and noradrenaline reuptake inhibitor, sibutramine, in the rat. *Br. J. Pharmacol.*, **121**, 1613–1618.

JACKSON, H.C., NEEDHAM, A.M., HUTCHINS, L.J., MAZURKIEWICZ, S.E. & HEAL, D.J. (1997a). Comparison of the effects of sibutramine and other monoamine reuptake inhibitors on food intake in the rat. *Br. J. Pharmacol.*, **121**, 1758–1762.

JUNG, R.T. (1997). Obesity and nutritional factors in the pathogenesis of non-insulin dependent diabetes mellitus. In *Textbook of Diabetes*. eds. Pickup J.C. & Williams G. Blackwell Science: Oxford.

LAMBERT, P.D., WILDING, J.P.H., TURTON, M.D., GILBEY, S.G., GHATEI, M.A., BLOOM, S.R. (1994). Effect of food deprivation and streptozotocin-induced diabetes on hypothalamic neuropeptide Y release as measured by a radioimmunoassay-linked microdialysis procedure. *Brain Res*, **656**, 135–140.

LE FEUVRE, R.A., AISENTHAL, L. & ROTHWELL, N.J. (1991). Involvement of corticotrophin releasing factor (CRF) in the thermogenic and anorexic actions of serotonin (5-HT) and related compounds. *Brain Res.*, **555**, 245–250.

LEVIN, B.E. (1990). Obesity-prone and -resistant rats differ in their brain [3H]paraminoclonidine binding. *Brain Res.*, **512**, 54–59.

LEVIN, B.E. (1991). Defective cerebral glucose utilization in diet-induced obese rats. *Am. J. Physiol.*, **261**, R787–R792.

LEVIN, B.E. (1999). Arcuate NPY neurons and energy homeostasis in diet-induced obese and resistant rats. *Am. J. Physiol.*, **276**, R382–R387.

LEVIN, B.E. & SULLIVAN, A.C. (1989). Differences in saccharin-induced cerebral glucose utilization between obesity-prone and -resistant rats. *Brain Res.*, **488**, 221–232.

LEVINE, A.S., WELDON, D.T., GRACE, M., CLEARY, J.P. & BILLINGTON, C.J. (1995). Naloxone blocks that portion of feeding driven by sweet taste in food-restricted rats. *Am. J. Physiol.*, **268**, R248–R252.

LEWIS, D.E., SHELLARD, L., KOESLAG, D.G., BOER, D.E., MCCARTHY, H.D., MCKIBBIN, P.E., RUSSELL, J.C. & WILLIAMS, G. (1993). Intense exercise and food restriction cause similar hypothalamic neuropeptide Y increases in rats. *Am. J. Physiol.*, **264**, E279–E284.

MAFFEI, M., HALAAS, J., RAVUSSIN, E., PRATLEY, R.E., LEE, G.H., ZHANG, Y., FEI, H., KIM, S., LALLONE, R., RANGANATHAN, S. KERN, P.A. & FRIEDMAN, J.M. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat. Med.*, **1**, 1155–1161.

MCNEELY, W. & GOA, K.L. (1998). Sibutramine. A review of its contribution to the management of obesity. *Drugs*, **56**, 1093–1124.

MORRIS, B.J. (1989). Neuronal localisation of neuropeptide Y gene expression in rat brain. *J. Comp. Neurol.*, **290**, 358–368.

PELLETIER, G. (1990). Ultrastructural localization of neuropeptide Y in the hypothalamus. *Ann. N. Y. Acad. Sci.*, **611**, 232–246.

ROGERS, P., MCKIBBIN, P.E. & WILLIAMS, G. (1991). Acute fenfluramine administration reduces neuropeptide Y concentrations in specific hypothalamic regions of the rat: possible implications for the anorectic effect of fenfluramine. *Peptides*, **12**, 251–255.

SAWCHENKO, P.E., SWANSON, L.W., GRZANNA, R., HOWE, P.R., BLOOM, S.R. & POLAK, J.M. (1985). Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. *J. Comp. Neurol.*, **241**, 138–153.

SCHWARTZ, M.W., SIPOLS, A.J., MARKS, J.L., SANACORA, G., WHITE, J.D., SCHEURINK, A., KAHN, S.E., BASKIN, D.G., WOODS, S.C., FIGLEWICZ, D.P. & PORTE, D. (1992). Inhibition of hypothalamic neuropeptide Y gene expression by insulin. *Endocrinology*, **130**, 3608–3616.

SOLIN, M.S., BALL, M.J., ROBERTSON, I., DE SILVA, A., PASCO, J.A., KOTOWICZ, M.A., NICHOLSON, G.C. & COLLIER, G.R. (1997). Relationship of serum leptin to total and truncal body fat. *Clin. Sci. (Colch.)*, **93**, 581–584.

STANLEY, B.G., KYRKOULI, S.E., LAMPERT, S. & LEIBOWITZ, S.F. (1986). Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides*, **7**, 1189–1192.

STOCK, M.J. (1997). Sibutramine: a review of the pharmacology of a novel anti-obesity agent. *Int. J. Obes. Relat. Metab. Disord.*, **21** (Suppl 1): S25–S29.

STOCK, M.J. & ROTHWELL, N.J. (1986). The role of brown fat in diet-induced thermogenesis. *Int. J. Vitam. Nutr. Res.*, **56**, 205–210.

STRICKER-KONGRAD, A., SOUQUET, A.M. & BURLET, C. (1995). Effects of sibutramine on feeding behaviour in obese and lean Zucker rats. *Int. J. Obes.*, **19** (Suppl 2): 398.

TRAYHURN, P. & DUNCAN, J.S. (1994). Rapid chemiluminescent detection of the mRNA for uncoupling protein in brown adipose tissue by northern hybridization with a 32-mer oligonucleotide end-labelled with digoxigenin. *Int. J. Obes. Relat. Metab. Disord.*, **18**, 449–452.

TRAYHURN, P., DUNCAN, J.S. & RAYNER, D.V. (1995). Acute cold-induced suppression of ob (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system. *Biochem. J.*, **311**, 729–733.

VUAGNAT, B.A., PIERROZ, D.D., LALAOUI, M., ENGLARO, P., PRALONG, F.P., BLUM, W.F. & AUBERT, M.L. (1998). Evidence for a leptin-neuropeptide Y axis for the regulation of growth hormone secretion in the rat. *Neuroendocrinology*, **67**, 291–300.

WALKER, H.C. & ROMSOS, D.R. (1993). Similar effects of NPY on energy metabolism and on plasma insulin in adrenalectomized ob/ob and lean mice. *Am. J. Physiol.*, **264**, E226–E230.

WANG, Q., BING, C., AL-BARAZANJI, K. MOSSAKOWASKA, D.E., WANG, X-M., MCBAY, D.L., NEVILLE, W.A., TADDAYON, M., PICKAVANCE, L., DRYDEN, S., THOMAS, M.E.A., MCHALE, M.T., GLOYER, I.S., WILSON, S., BUCKINGHAM, R., ARCH, J.R.S., TRAYHURN, P. & WILLIAMS, G. (1997). Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. *Diabetes*, **46**, 335–341.

WILDING, J.P., GILBEY, S.G., JONES, P.M., MANNAN, M.M., GHATEI, M.A. & BLOOM, S.R. (1992a). Dexfenfluramine treatment and hypothalamic neuropeptides in diet- induced obesity in rats. *Peptides*, **13**, 557–563.

WILDING, J.P., GILBEY, S.G., MANNAN, M., ASLAM, N., GHATEI, M.A. & BLOOM, S.R. (1992b). Increased neuropeptide Y content in individual hypothalamic nuclei, but not neuropeptide Y mRNA, in diet-induced obesity in rats. *J. Endocrinol.*, **132**, 299–304.

WILLIAMS, G., HARROLD, J.A. & CUTLER, D.J. (2000). The hypothalamus and the regulation of energy homeostasis: lifting the lid on a black box. *Proc. Nut. Soc.*, **59**, 385–396.

WILMOT, C.A., SULLIVAN, A.C. & LEVIN, B.E. (1988). Effects of diet and obesity on brain alpha 1- and alpha 2-noradrenergic receptors in the rat. *Brain. Res.*, **453**, 157–166.

ZARJEVSKI, N., CUSIN, I., VETTOR, R., ROHNER-JEANRENAUD, F. & JEANRENAUD, B. (1994). Intracerebroventricular administration of neuropeptide Y to normal rats has divergent effects on glucose utilization by adipose tissue and skeletal muscle. *Diabetes*, **43**, 764–769.

(Received November 13, 2000
Revised December 5, 2000
Accepted February 12, 2001)